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Does density influence relative growth performance of farmed, wild, and F1 hybrid Atlantic salmon in semi-natural and hatchery common garden conditions?

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ABSTRACT

The conditions encountered by Atlantic salmon, *Salmo salar* L., in aquaculture are markedly different from the natural environment. Typically, farmed salmon experience much higher densities than wild individuals, and may therefore have adapted to living in high densities. Previous studies have demonstrated that farmed salmon typically outgrow wild salmon by large ratios in the hatchery, but these differences are much less pronounced in the wild. Such divergence in growth may be explained partly by the offspring of wild salmon experiencing higher stress and thus lower growth when compared under high density farming conditions. Here, growth of farmed, wild and F1 hybrid salmon were studied at contrasting densities within a hatchery and semi-natural environment. Farmed salmon significantly outgrew hybrid and wild salmon in all treatments. Importantly however, the reaction norms were similar across treatments for all groups. Thus, the present study was unable to find evidence that the offspring of farmed salmon have adapted more readily to higher fish densities than wild salmon as a result of domestication. It is suggested that the substantially higher growth rate of farmed salmon observed in the hatchery compared to wild individuals may not solely be caused by differences in their ability to grow in high density hatchery scenarios.

Key words: Density, Domestication, Farm escapes, Genetic interaction, Growth, Hybridisation,

INTRODUCTION

Captive populations undergo various morphological, physiological and behavioural changes during domestication (1). Adaptation to the domestic environment occurs through two routes: environmentally induced changes to developmental processes within a single generation and genetic change across generations (2,3). Relaxed natural selection can also result in domestic individuals that are more variable than wild conspecifics for certain traits which have adaptive value in the wild but less so in captivity (4). For example, low mortality associated with domestic environments results in phenotypes persisting where they would not have persisted in the wild (5,6). Genetic and morphological change occurs through direct and indirect responses to artificial selection and natural

selection within the domestic environment contrasted with the wild environment (local adaptation in wild populations), and the differential mortality described above (2,5,6). Random changes in allele frequencies can also arise through genetic drift in domestic populations with limited effective population sizes (4). Thus, many domestic populations have become adapted to their captive environment, and may have reduced fitness in natural or novel environments when compared to wild individuals (3,4). A loss of adaptive potential through domestication can negatively influence wild populations if domesticated individuals interbreed with wild conspecifics, such as when farmed individuals are released for restocking or are accidentally released through escape events.

Domesticated fish experience environments which differ vastly from those in nature in several ways (5,6). Compared to the wild, hatchery environments typically display reduced environmental variation, fish densities are much higher, food is provided in excess, predation is absent, and there is no competition for mates (7,8). Furthermore, there is often strong directional selection for a variety of commercially valuable traits such as growth rate and delayed maturation (9,10). The outcome is that domestic fish are different to wild fish for several behavioural, morphological and physiological traits (5), likely underlain by genetically-based as well as phenotypic plasticity (8).

Atlantic salmon (*Salmo salar*, Linnaeus (1758)) are iteroparous fish native to rivers on the east and west coasts of the Atlantic Ocean in the Northern hemisphere (11). They typically display an anadromous life cycle, although some populations spend their entire life cycle in freshwater. Stream-dwelling populations of wild Atlantic salmon typically exhibit territoriality (12), and individual growth and survival are regulated through exploitative (indirect competition for communal resources) and interference (direct resource competition through dominance or fighting) competition (13). The density of salmon tends to vary greatly among and within river systems (14). When densities are high, competition is exacerbated and the population is regulated by density-dependent mortality, emigration or displacement (12). Less commonly the territory size of an individual will decrease, causing individual growth to decrease. Thus, population regulation occurs through density dependent growth (13), though this type of population regulation is more common in lake-dwelling fish where

emigration is not possible (12). Studies show that when density in the wild is increased, individual growth decreases due to density-dependent factors (12,15).

Growth is an important component of fitness (8), and body size is known to influence the outcome of social and resource competition (13,16). Farmed Atlantic salmon have been under direct selection for fast growth for more than ten generations, and consequently the offspring of farmed salmon typically outgrow wild salmon by up to several fold under communal hatchery conditions (17-20). In the wild, however, growth differences are far less pronounced (21-23). The lower growth and survival of farmed fish within wild environments may be due to the high metabolic costs associated with increased aggression or maladapted foraging behaviour of farmed escapees (5), or their inability to adapt to variable feed in the natural environment (24). Conversely high growth differences observed between farmed and wild fish in the hatchery might derive from adaptation of farmed salmon to high densities, typically fed to excess. Reduced response to stress relative to their wild conspecifics has been documented in domestic salmon (19) and sea trout (anadromous *Salmo trutta* L.) (25). While the increased stress, competition and social interaction associated with high densities would intuitively result in decreased growth as described above, it is thus possible that the domestication process has resulted in farmed strains that maintain high growth at high densities.

Understanding how changing environmental conditions such as density affect growth and survival in domestic and wild conspecifics, and their hybrids, can increase our knowledge of the risks associated with escapees of farmed fish and the consequences of hybridisation. Here, a common garden design was used to investigate the growth of farmed, wild and F1 hybrid Atlantic salmon offspring at three contrasting densities within a hatchery, and at two contrasting densities under semi-natural conditions. The aim was to investigate whether differences in growth rates between farmed, wild and F1 hybrid salmon displayed similar reaction norms at different densities in the two environments. Specifically, the hypothesis tested was that the relative growth difference between farmed and wild salmon would be higher in the high density conditions as a result of adaptation of farmed salmon to those conditions.

MATERIALS AND METHODS

Family Production

All families used in this experiment were established in November 2013 at Matre, the Institute of Marine Research's (IMR) experimental fish-farm in Norway. Atlantic salmon from the commercial farmed strain Mowi and wild caught Atlantic salmon from the River Etne (59°40'N, 5°56'E) were used to produce five pure farmed, five pure wild, and five F1 hybrid families (15 families in total) (Table S1). Mowi is the oldest Norwegian commercial strain and is used by Marine Harvest (26). Mowi was established in the late 1960s primarily using fish from the River Vosso and the River Aaroy, whose populations are known to contain large multi-sea winter fish (18). The main traits that have been under selection in the Mowi strain are growth, late maturation and fillet quality. The farmed salmon used in this study had undergone over 10 generations of selection. The salmon population from the River Etne is the largest salmon population in Hordaland, western Norway. A 2004 report estimated that the smolt production for the River Etne was around 30 000 individuals in a 15km area (27). A study conducted using snorkelling observations and catch statistics for the period 2004-2011 estimated that the median number of wild fish in the Hardangerfjord river system (including the River Etne) was estimated to be 3.5 fish per 10 000m² using (28). The wild parental salmon were collected directly from the river in the autumn of 2013 by angling and transferred to the local hatchery where they were held until gametes were stripped from the fish. Fish scales were read from these individuals in order to ensure that they were wild fish and not farmed escapes (29). Population genetic analyses have revealed introgression of farmed salmon in a number of Norwegian populations, including the population in the river Etne (30,31). Therefore, although the wild fish used in this study were indeed born in the wild (based upon scale reading), it is not possible to completely exclude the possibility that some of those individuals used as broodstock may have admixed ancestries at some level.

All F1 hybrids were produced by crossing a farmed Mowi female with a wild Etne male (Mowi x Etne). The hybrids were thus maternal and paternal half-siblings with the farmed and wild families, respectively. From here on group refers to the origin of each cross-type, *i.e.* farmed, wild and hybrid.

Eyed eggs were sorted into hatchery trays representing the treatment replicates in week 5 of 2014 (where week 1 = first week of January). The replicates were all incubated under standard hatchery conditions until transfer to tanks. Dead eggs were removed when necessary. In the hatchery treatments, the control and high density replicates initially consisted of 30 eggs each from the 15 families ($n = 450$ per tank) while each low density replicate consisted initially of 15 eggs each family ($n = 225$ per tank). In the semi-natural treatments the low density replicates consisted of 30 eggs from each family ($n = 450$ per tank) and the high density replicates consisted of 90 eggs per family ($n = 1350$ per tank). Egg volume measurements were taken from each family in order to calculate average family egg diameter. Egg diameter was calculated as 25cm divided by the number of eggs counted on a 25cm rule.

Experimental Design

In order to investigate the effect of density and environment on growth and survival in salmon of farmed, wild and hybrid origin, fish were reared in communal fish tanks (*i.e.*, common garden) at three densities in a hatchery environment and at two densities in a semi-natural environment. These treatment densities were chosen to represent densities that farmed and wild fish may not typically experience in their respective local environments, where typically the farming environment is characterised by much higher densities than the wild environment. For an overview of the experiment see Table 1. The treatments consisted of five differing rearing conditions: three hatchery treatments further differentiated into high, low, and control densities, and two semi-natural treatments consisting of high and low densities. Treatment from here on refers to the five different rearing conditions as described below.

Hatchery treatments

Three treatments were set-up within a hatchery environment to represent (i) low density (~ 0.16 fish/L) (ii) a control density (~ 0.36 fish/L) which represented a standard hatchery density (iii) a high density environment (~ 1.5 fish/L). These are hereon referred to as the low, control, and high hatchery treatments. Each treatment consisted of two replicate tanks with 6 experimental hatchery tanks in total. The low density treatment was established by initially using half the number of fish used in the control and high treatments. The high density treatment consisted of the same initial number of fish as the control treatment with a water level 25% of the control water level (55cm) to simulate a high density environment.

Unfed-fry were transferred from the hatchery incubators to the experimental tanks in week 17, when treatment conditions commenced. The fish were reared in 1.5m^2 tanks with a maximum flow rate of 35L/min at ambient water temperature. Temperature was recorded daily and ranged from 4.5 to 14.4°C . Start feeding began in week 18, and fish were fed a commercial pellet diet (Skretting) *ad libitum*. Pellet size was adjusted according to manufacturer's tables, and the fish were kept on 24 hour photoperiod throughout the experiment as is standard in salmon hatcheries.

Semi-natural treatments

The semi-natural environment consisted of replicate donut-shaped 7.85m^3 tanks (outer diameter 7m, inner diameter 3m) filled with gravel (of variable sizes to reflect a natural river bed, no larger than $\sim 30\text{cm}$ in diameter) and situated outdoors (20,32). Water level was kept at 25cm in both treatments.

The density conditions were imposed by adding three times as many fish into the high density treatment (~ 0.11 fish/L) compared to the low density treatment (~ 0.05 fish/L). The treatments are from here on referred to as the low and high semi-natural treatments. Each treatment consisted of two replicate tanks; therefore there were 4 experimental semi-natural tanks.

Fish were planted out as fry into the semi-natural environment in week 17, when treatment conditions commenced. Automatic feeders were situated near the water inlet and fish were fed *ad libitum* as in the hatchery experiment. The fish experienced natural light conditions and ambient water temperature

which ranged from 4.6 to 14.4°C across the experimental period (Supplementary Figure S1). Average daily temperature was used to calculate the degree days for the hatchery and semi-natural treatments. The semi-natural tanks were predator-free, *i.e.* no predators were explicitly placed within the tanks.

Sampling & Data

The experiment ran for 20 weeks and was terminated in calendar week 37 of 2014. Mortality was recorded daily for each hatchery treatment replicate and was used to estimate total mortality at experiment termination. Average biomass within the hatchery treatments was estimated each month by measuring 100 randomly sampled fish in each replicate, which allowed for the estimation of stocking density within these treatments as the experiment progressed (Figure 1). It was not possible to record daily mortality in the outdoor semi-natural tanks, however total mortality was estimated using the number of surviving fish sampled at the end of the experiment. Mortality data are presented in Table 2. All remaining fish were euthanized with an overdose of Finquel Vet anaesthetic following standard guidelines (Årnes, Norway). Individual growth measurements of wet weight and fork length were recorded and adipose or caudal fin samples were taken from each individual and stored in 100% ethanol. A total of 2105 individuals were sampled in the hatchery tanks and a total of 1883 individuals were sampled in the semi-natural tanks.

Genotyping and Parentage Assignment

DNA-based parentage testing was used to assign individual fish from the hatchery and semi-natural treatments respectively back to their family of origin. DNA was extracted in 96 well plates using a variation of the salt extraction method (33). Parental DNA was extracted and genotyped twice to ensure consistent genotyping. Each plate contained 2 randomly placed negative controls (blank wells) to ensure unique identification of each plate. Five microsatellites were amplified in a single PCR multiplex: *SsaF43* (34), *Ssa197* (35), *SSsp3016* (Genbank # AY372820), *MHCI* (36), and *MHCII* (37). There were 38 individuals from the hatchery experiment and 82 individuals from the semi-natural experiment which could not be unambiguously assigned back to one family using the original

multiplex. These samples were genotyped using additional loci (Supplementary Table S2) in order to unequivocally identify their families. PCR products were resolved on an ABI Applied Biosystems 3731 Genetic Analyser and sized using a 500LIZ standard (Applied Biosystems). Genemapper Version 4.0 was used to score alleles manually. Individuals were then assigned back to family using the Family Analysis Program (FAP) (v3.6) (38).

Statistical Analysis

Statistical analysis was carried out using R version 3.1.3 (39), and all critical p-values were set to 0.05 unless otherwise stated.

Growth

A linear mixed effect model (LME) was used to investigate the variation in weight at termination. The response variable was the continuous variable of log-transformed weight at termination. The LME model was fitted using *lmer* from the *lmerTest* package in R (40). The full model was fitted with treatment (T) and group (G) as fixed categorical factors, egg size (E) as a continuous fixed effect, and all two-way interactions between the fixed covariates: treatment and group (TG); treatment and egg (TE); and group and egg (GE) as fixed effects. Tank replicates (t) nested within treatments were included as a random intercept effect (10 levels), and family (f) was included as a random intercept effect (15 levels) with differing slopes for the effect of treatment:

$$Y = \beta_0 + \beta_1 T + \beta_2 G + \beta_3 E + \beta_4 TG + \beta_5 TE + \beta_6 GE + b_t + b_f + \varepsilon \text{ where } \varepsilon \sim N(0, \sigma^2) \quad (2.1)$$

where β_0 is the intercept and ε is the normally distributed error term. The *lmerTest* package in R allows for automatic model selection using the *step* function (40). The function performs backwards selection on both the fixed and random effects to determine the simplest best-fitting model (40):

$$Y = \beta_0 + \beta_1 T + \beta_2 G + \beta_3 E + \beta_4 TE + b_t + b_f + \varepsilon \text{ where } \varepsilon \sim N(0, \sigma^2) \quad (2.2)$$

It first performs backwards selection on the random elements of the model using likelihood ratio tests, with a significance level of 0.1 as a default, before performing backwards selection on the fixed

elements in the model (40). The p-values generated for the fixed part of the model are calculated using an F-test based on Sattethwaite's approximation and the significance level is set to 0.05 (40). Both the full and final model fits were confirmed by investigating plots of the model residuals against the covariates included in the model as well as those which were not included in the model. Normality of the model residuals was confirmed visually using histograms. The full and final model with parameter estimates as given by the *lme4* output with overall covariate p values generated from the *step* function is presented in Table 3. Pair-wise comparisons of log weight between treatments and between groups were performed using the *glht* function in the *multcomp* package with Tukey adjustments for multiple comparisons (Table S3) (41). Pair-wise comparisons of egg size among the groups were performed using the *glht* function as above (Table S3). Relative growth differences comparing the average weight in grams and log weight of farmed to wild and hybrid to wild fish are presented for each treatment in Table 4.

Mortality

In order to investigate whether survival differed between treatments, a generalized linear mixed effect model (GLMM) was fitted using the *glmer* function in the *lme4* package (42). The full model included the fixed factor covariates of treatment (T) and group (G), the continuous effect of egg size (E), and two-way interactions between the fixed covariates: treatment and egg (TE), treatment and group (TG) and group and egg size (GE). In order to control for any differences in mortality between replicates and families the variables tank (t) and family (f) were included in the model as random intercept covariates:

$$\text{logit}(Y) = \beta_0 + \beta_1 T + \beta_2 G + \beta_3 E + \beta_4 TE + \beta_5 TG + \beta_6 GE + b_t + b_f + \varepsilon \quad (2.3)$$

where β_0 is the intercept and ε is the error term. The response variable, survival, was binary, and thus the binomial distribution was used with the default logit link function and the model was fitted using the Laplace approximation. The random effect structure was investigated by fitting the full model with only one random effect at a time and plotting the 95% prediction intervals of the random effect

using the *dotplot* function in the lattice package (43). If all the prediction intervals of the random effect overlapped zero then this effect was removed from the final model. The mixed function from the afex package was used to investigate the significance of the fixed covariates (44). The function calculates type 3-like p-values for each fixed covariate based on parametric bootstrapping (44). Parameter estimates and the p –values of the fixed effects are presented in Table 5. The final model included covariates which yielded the best fit:

$$\text{logit}(Y) = \beta_0 + \beta_1T + \beta_4TE + b_f + \varepsilon \quad (2.4)$$

RESULTS

Genotyping and Parentage Assignment

Of the 3988 individuals sampled, 11 individuals (<0.001% of the total) could not be assigned unambiguously back to a single family using the microsatellite multiplexes. A further 4 individuals were identified as outliers due to extreme condition factors attributed to human recording error and subsequently removed from the dataset prior to analysis. Thus, a total of 3973 individuals were used in the analysis.

Statistical Analysis

Growth

Treatment, group, egg size and the interaction of egg size and treatment were retained as significant effects in the growth model (Table 3). All genetic groups grew significantly different to each other across the treatments, with farmed fish being larger than hybrid and wild fish, and hybrid fish being larger than wild fish (Supplementary Table S3; Figure 2). On average, all fish grew larger in the hatchery density treatments and growth of all groups was lowest in the semi-natural density treatments (Figure 2). The interaction between treatment and group was not significant, indicating that all groups responded equally relative to the other groups across the treatments, indicated by the

similar relative growth differences in Table 4 and the reaction norms in Figure 3. Within the hatchery treatments, growth of all three genetic groups decreased as density increased, with the lowest growth observed in the high density hatchery treatment, although the difference in growth between the hatchery treatments was not significant (Supplementary Table S3). Similarly growth was not significantly different between the two semi-natural treatments, although it was visibly lowest in the semi-natural high density treatment (Figure 2). The final model (Equation 2.2, Table 3) retained an effect of egg size and a significant interaction between egg size and treatment. Egg size was significantly different among the groups (Supplementary Table S3) and was found to be negatively correlated to weight. It was found that egg size was only a significant predictor of weight in the semi-natural treatments, as the fish in these treatments displayed the lowest weights, possibly due to a slower development compared to the hatchery treatments (Supplementary Table S4). There was a difference in degree days between the hatchery (1796 degree days) and the semi-natural treatments (1586 degree days) due to different ambient temperatures between the indoor (hatchery) and outdoor (semi-natural) tanks (Supplementary Figure S1). Egg size was also significant in the hatchery high density treatment, where growth was also low. The random effects of tank replicate and family were retained in the final model in order to control for any variation within these variables.

Mortality

Percentage survival was highest in the hatchery treatments, with no significant differences among treatments observed (Table 2, Figure 4). Within the semi-natural treatments for all groups, survival was highest in the high density treatment (Table 2, Figure 4). The low survival observed in the semi-natural low density treatment was not a result of high mortality in one specific replicate: the random effect of tank was excluded from the final model due to its non-significant effect; therefore mortality was insignificantly different between replicates within each treatment. The final model retained a significant effect of treatment and an interaction between egg size and treatment, while egg size alone was not significant (Equation 2.4, Table 5). On further analysis of the data split into each treatment, it was found that egg size was only significant in the hatchery high density treatment (Supp table 5).

DISCUSSION

Growth and survival of fish is influenced by density and availability of food (45,46). The offspring of farmed Atlantic salmon generally outgrow wild salmon two-fold or more under hatchery conditions (18-20), possibly due to adaptation to high densities through domestication. Therefore, it was hypothesised that farmed salmon may be able to maintain higher growth than their wild conspecifics in high density environments, potentially explaining the elevated growth differences observed between farmed and wild conspecifics under hatchery conditions. Here, it was found that density influenced growth of all genetic groups equally, with all groups exhibiting decreased growth at higher densities; farmed salmon had the highest average growth within each treatment while wild fish had the lowest growth within each treatment; and the mortality of all groups was similar for all the treatments. Thus the present study was unable to find evidence of adaptation of farmed fish to high densities using the present treatment densities, tentatively suggesting that high density adaptation is not solely driving the divergence in growth observed between farmed and wild salmon under hatchery conditions.

Growth

High density conditions are known to lead to behavioural changes, induce stress behaviours and lower feed utilisation, all of which can decrease growth among fish (47). Refstie & Kittelsen Bohlin (45) found that under controlled conditions with excess feed the growth of two domesticated populations of Atlantic salmon decreased as density increased. The negative effect of higher densities on growth has also been observed in other fish species (15,48), and has been attributed to an increase in intraspecific competition and agonistic behaviour at high densities (12,49). In natural systems density dependent growth will also be controlled by the number of predators and by the competition for limited resources (13). As there were no predators in the present study, and the available food was not limiting, it is likely that the lower growth observed at the higher densities could be the result of higher crowding stress in all groups which may have caused the fish to feed less effectively relative to the other treatments..

Growth and body size is an important factor determining competition and reproductive success in fishes (50). Directional selection for growth has resulted in farmed salmon displaying higher growth rates than wild salmon when compared under hatchery conditions (19), and this growth may give the offspring of escaped farmed salmon a competitive advantage over wild conspecifics in the wild, although often the growth differences observed in the wild are much lower (21,22). Under standard hatchery conditions the relative growth differences between farmed and wild conspecifics has been documented to be as high as 3-fold (19) and even 5-fold (20), with Glover et al (18) observing that farmed salmon were twice as large as their wild conspecifics at the end of a full aquaculture production cycle. In the present study farmed salmon grew significantly larger than wild salmon in all treatments, although this growth difference was much lower than previously observed in a hatchery study using the same strains (19). Interestingly, Reed et al (23) reported relatively moderate differences (5-20%) between farmed and wild salmon parr for size-at-age in the wild, and they found that their observed growth differences were similar in the hatchery environment as in the wild, contrasting the results of previously cited studies. They attribute these differences to the difference in historical selection regimes and generation time between the farmed strain used in their study (Irish farm strain derived from the Norwegian Mowi strain in 1983) compared to the other studies (more recent Norwegian Mowi strain) (23).

Solberg et al (19) found that juvenile farmed salmon exhibited a lower response to stress than their hybrid and wild conspecifics when exposed to a twice-daily stressor of lowered water levels, indicating that domestication has resulted in farmed salmon which are able to maintain a higher level of growth under stressful conditions. Elevated stress due to crowded conditions has been shown to negatively influence appetite and growth performance in Atlantic salmon (51) and brown trout (52). It is possible that the process of domestication may have adapted farmed salmon to higher growth under stressful high density conditions. Thus, farmed salmon in the present study would be expected to maintain a higher growth relative to the wild salmon at high densities within the hatchery treatment due to a relaxed response to crowding stress. However, this was not the case here. No evidence was found for an interaction between group and treatment (genotype by environment interaction) (Table

3), and the similar relative growth differences between groups among the treatments indicate that each group is responding to the treatments similarly relative to the other groups (Table 4, Figure 3). It is acknowledged that the treatments used in the present study may not have been different enough to elicit a growth divergence response due to density adaptation and that such adaptations may manifest at higher densities; however the findings suggest that the higher growth differences observed in the hatchery are probably solely not the result of farmed fish being more adapted to growth at higher densities than wild fish.

The ability of an individual to adapt its behavioural strategy (plasticity) can influence fitness and competition (53). Many salmonids exhibit behavioural plasticity depending on the circumstance, for example exhibiting territorial behaviour in low densities, and schooling behaviour in high densities (53,54). At certain densities it becomes too metabolically costly to defend a territory (53,54). Under controlled conditions Brännäs et al (53) found that interspecific competition among stocked brown trout depended on a variety of factors including competitive ability, food availability and prior residency. They found that growth of all groups was depressed at higher densities and it was advantageous to be less aggressive at high densities and also to be a larger individual (53). Farmed salmon are generally observed to be more aggressive than wild salmon, possibly inadvertently through selection for increased growth or because they have not been able to establish social or dominance hierarchies under hatchery conditions and may not understand the trade-off between aggression and its energetic cost in certain situations (5). Higher levels of growth hormone (GH) may also influence aggression in salmonids (55), and may also affect foraging behaviour and metabolic demands (16). These behavioural and hormonal changes within farmed salmon may partly explain their lower relative growth observed in the wild. Solberg et al (20) found that growth differences between farmed and wild conspecifics decreased along an environmental gradient from hatchery to semi-natural conditions with restricted feed. They suggest that the lower growth observed in wild studies could be caused by a combination of negative and positive size-selective mortality, whereby faster growing individuals can outcompete smaller individuals for resources (negative size selection) and where faster growing individuals are more prone to predation over smaller individuals (positive

size selection), resulting in fish of all origins being of a similar size (although positive size selection was not explicitly tested in their study) (20).

In the present study growth was low among all groups in the semi-natural treatments (Figure 2), despite these two treatments having the lowest densities among all treatments. In the wild, salmonids are territorial and establish a social hierarchy among individuals which influence individual growth, with low-ranking fish having reduced access to feed and displaying reduced growth relative to the dominant individuals (49). If the semi-natural environment induced territorial or dominance effects among the fish, one would then expect to see distinct size classes representing the larger, dominant fish, and the smaller, less dominant individuals. However, such trends were not observed. There was a difference in degree days between the hatchery and semi-natural treatments; therefore it is therefore likely that other environmental conditions such as the naturally varying water temperature or ambient light conditions were responsible at least partly for the low growth observed in the semi-natural treatments. It is possible that the densities imposed on these semi-natural tanks were not sufficient to affect growth divergence among the groups. Jørgensen et al (49) investigated the effects of density on hatchery-reared Arctic charr (*Salvelinus alpinus* L.) under controlled conditions. Interestingly, they found depressed growth rates in the low density treatment, and observed schooling behaviour of fish in their medium and high density tanks (49). In the present study schooling behaviour was observed within the high density semi-natural replicates. While the low water temperature is probably the main reason behind the low growth observed in the semi-natural treatments, it is possible that the increased swimming behaviour and social interaction may have influenced growth.

In similar comparative studies of Atlantic salmon, hybrids often display intermediate levels of growth compared to their farmed and wild parental strains (18,20,55,56). Hybrid vigour commonly occurs when one or both of the parental strains are inbred, whereas a decreased performance observed in hybrids relative to their parents may occur via outbreeding depression (55). In the present study hybrids grew significantly different to both wild and farmed conspecifics, however there was an observable non-significant trend of hybrid relative growth being more similar to their farmed parents

in each treatment (1.5-1.6:1 for hybrid to wild and 1.2-1.3:1 for farmed to hybrids using the average raw weight in grams) (Table 4, Figure 3). A study which used the same parental strains as the present study also observed intermediate hybrid growth and the same trend of more similar growth with the farmed parents (19). It is not thought that the growth levels observed in the present study represent hybrid vigour, as growth was significantly different among the groups (Supplementary Table S3) and the relative growth differences between the hybrids and their parental groups are still intermediate (Table 5, Figure 3), indicating additive effects. It is acknowledged, however, that a more complete hybrid group design (*i.e.* reciprocal crosses) would allow for the unambiguous conclusion of additive hybrid growth effects. Several studies comparing gene transcription between farmed and wild salmonids observed some level of non-intermediate (non-additive) gene expression in hybrids (57-59), and this may be population specific (60,61). Bicskei et al (58) examined gene transcription in farmed, F1 hybrid and wild Atlantic salmon at two life stages, and found fewer significantly differentially expressed transcripts between farmed and hybrid individuals than between hybrid and wild individuals. Their hybrid crosses were generated from the farmed females, and suggest that maternal effects might account for this bias (58). They found that the heritability patterns of many of the differentially expressed transcripts in the hybrid fish were either intermediate or maternally dominant (58), highlighting the need for reciprocal hybrid crosses in comparative studies. Maternal effects, such as egg size or maternal body size, can greatly influence offspring development and fitness (62). Often maternal effects are taken into account in order to avoid overestimating or confusing genetic effects with environmental maternal effects (63). In the present study, the maternal effect of egg size was controlled for by including it as a covariate in the growth model.

Overall, egg size was found to be negatively influencing growth, due to the larger average egg sizes of the wild families used in the present study coupled with their lower growth compared to the farmed and hybrid families. Generally, a larger egg size is expected to convey a positive size advantage to offspring (64), however negative maternal effects have been observed in Chinook salmon (*Oncorhynchus tshawytscha* L.), whereby the initial positive effect of large egg size on growth was reversed after a period of time (63). The authors attribute this switch in egg size effect to variation in

growth rate among families with different egg sizes (63). In the present study the growth model identified the interaction between egg size and treatment as a significant predictor of growth (Equation 2.2, Table 4), and when egg size was included in the models for growth at each treatment, it was found that it was only significant in the semi-natural treatments and the hatchery high density treatment. It is possible that the lack of degree days in meant the smaller fish had had less time to develop and had not yet overcome the effect of egg size, which is known to decrease with offspring development (65).

Mortality

Mortality within the hatchery treatments was low, and did not differ between treatments or between the groups (Table 5, Figure 4). There was high mortality observed within the low density semi-natural replicates (81.2 and 78.3 %, Table 2), and moderate mortality within the high density semi-natural tanks (36.3 and 37.9 %, Table 2). It is not possible to determine when the majority of this mortality occurred, or whether it was a gradual or acute event. It is therefore not possible to say how this may have influenced growth as the experiment continued. In natural conditions salmonids are territorial (13) and this may impose a density dependent effect on mortality. Within a stream environment as population density increases past the carrying capacity for territories several processes can occur: territory size may decrease and influence growth through density dependence or those who are unable to acquire a territory and access to food may emigrate or die (12,13). Generally, mortality is observed to be positively related to stocking density (49), therefore it is unclear why it was the low density semi-natural replicates which suffered such high mortalities. There was no effect of group origin on mortality, indicating that all groups suffered similar relative mortalities (Figure 4). Interestingly, both replicates from each of the semi-natural treatments experienced similar mortality, indicating no influence of tank effects on mortality (Equation 2.4, Table 2). There was no observed predation from birds (I-H. Matre, pers. comm. 2014). The mortality model identified treatment and the interaction between treatment and egg size as predictors of survival (Equation 2.4, Table 5). When the effect of

egg size on mortality was investigated for each treatment, it was found that egg size was only significant within the high density hatchery treatment.

General Implications

While comparing the relative growth of farmed, hybrid and wild salmon families under different densities, there was no evidence found to suggest that farmed salmon have adapted to higher stocking densities. Although the possibility cannot be excluded that higher and lower densities than those used in this study may elicit such effects, our treatments nevertheless elicited a response in modifying growth of all salmon reared here. The lack of interaction between density and relative growth of farmed, hybrid and wild salmon observed here tentatively suggests that differences in relative growth between farmed, hybrid and wild salmon between the hatchery environment and the wild is caused by a complex of other factors, and not solely attributable mainly to density. Competitive experiments in the wild at differing densities have suggested that farmed salmon display relatively greater mortality than wild salmon under higher densities (22), and population genetic studies have demonstrated that the success of farmed salmon in the natural environment is also determined by native population density (30,31,66). It has been suggested that wild populations with lower densities (low population numbers) may be more at risk of the negative effects of hybridisation and introgression from farmed fish (31,66,67). Comparative studies within a natural setting are needed in order to further understand what drives the growth differences between wild and farmed salmon in the wild. Furthermore, comparative studies at more varied densities are encouraged in order to further elucidate the effects of density on growth differences between farmed and wild conspecifics.

Studies investigating the performance of hybrids are crucial for understanding how hybridisation between farmed and wild conspecifics influences wild population dynamics. Farmed escapees can successfully interbreed with wild salmon, producing F1 hybrid offspring, and the subsequent performance of these hybrids will likely determine the future success of the wild population (56).

Here, the hybrid growth was observably more similar to their farmed parents than their wild parents, which may influence their subsequent fitness in the wild. The hybrids in the present study were

maternal half siblings to the farmed fish; therefore it is possible that maternal effects influenced growth patterns. It is important therefore to understand how hybrids respond to changing environmental conditions for future salmonid conservation and management, and to include reciprocal hybrids in order to differentiate between the effect of maternal egg size and the effects of domestication. Further studies which investigate the performance of backcrosses and reciprocal hybrids with wild fish will further elucidate the impacts of introgression on local population fitness.

ETHICAL STATEMENT

The experimental (permit number 64472) was officially approved March 26 2014, by the Norwegian Animal Research Authority (NARA). All welfare and use of experimental animals was performed in strict accordance with the Norwegian Animal Welfare Act. In addition all personnel involved in this experiment had undergone training approved by the Norwegian Food Safety Authority, which is mandatory for all personnel running experiments involving animals included in the Animal Welfare Act.

DATA ACCESSIBILITY

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.2q5h1>

COMPETING INTEREST

We declare that we have no competing interest.

AUTHOR CONTRIBUTIONS

GRC, KG and MIT conceived the study. MS, LD and IHM performed the experiment. GJ and AH carried out the molecular lab work. MS participated in data analysis. AH performed the statistical analysis and wrote the manuscript with input from KG, MS, GRC, MIT, SC and GJ. All authors have read and approved the final manuscript.

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TABLE AND FIGURE CAPTIONS

Table 1: Details of the experimental design. Initial numbers of eggs per family within each replicate treatment and the water level and volumes of each treatment.

Table 2: Weight, mortality and average densities within treatments at experiment termination. High and low correspond to the density of fish in the treatments, while control represents an intermediate density. First and last correspond to the first density calculated from average biomass per treatment taken in week 23 and the final density measurement calculated from final weight data taken in week 37.

Table 3: Parameter estimates of the full model for the linear mixed model investigating log weight variation. The final model (equation 2.2) covariates are presented in bold. The final column gives single p values estimated for each covariate in the full model using the *step* function in the lmerTest package by an F-test based on Sattethwaite's approximation. The significance level is set to 0.05 unless otherwise stated. S.e.; standard error of the parameter estimates. S.d.; standard deviation of the variance estimates of the random effects.

Table 4: Relative weight and log weight differences between each group within each treatment. The relative growth differences were calculated by dividing the average weight (W) in grams of the farmed fish by the wild and hybrid fish respectively, and the average weight of the hybrid fish by the wild fish within each treatment. The relative log weight (Log W) differences were calculated as above using the average log weights (Log W) of each group within each treatment.

Table 5: Parameter estimates of the glmm model investigating variation in survival. Covariates in bold were retained in the final model (equation 2.4). The final column gives single p values estimated for each covariate within the final model estimated using the *mixed* function in the afex package by parametric bootstrapping. S.e.; standard error of the parameter estimates of the fixed effects. S.d.; standard deviation of the variance estimates of the random effects.

Figure 1: Average stocking density of the treatments. The stocking density was calculated by estimating average biomass per replicate by weighing a random sample of 100 fish from each tank at specific time points within the experiment duration. This was only possible for the hatchery tanks, and therefore only the stocking density at experiment termination is presented for the semi-natural tanks.

Figure 2: Average weights of each group within each treatment. Bars represent the standard error of the mean weight of each group within the treatments.

Figure 3: (a) Phenotypic growth reaction norms for each group across the treatments (average log weight) and (b) the average log weights relative to the wild group. In figure (b) the hybrid and farmed groups are compared to the wild group within each treatment. The x-axis shows the treatments.

Figure 4: Average number of fish surviving for each group within each treatment. Dotted horizontal lines represent the expected number of surviving fish per group in each treatment based on average mortality. Error bars represent the standard error of the average family variation per group within each treatment.

TABLES

Table 1: Details of the experimental design.

Treatment	Hatchery			Semi-natural	
	Low	Control	High	Low	High
Replicates (n)	2	2	2	2	2
Initial fish per replicate	225	450	450	450	1350
Families per replicate	5 farmed : 5 hybrid : 5 wild in all treatment replicate tanks				
Total fish	450	900	900	900	2700
Water level	55cm	55cm	13.5cm	25cm	25cm
Volume (m ³)	1.2375	1.2375	0.30375	7.85	7.85

Table 2: Weight and mortality within treatments at experiment termination.

Treatment	Tank	N - sampled	Weight (g)		Density (kg/1000L)		Mortality (%)
			Mean	SD	First	Last	
Hatchery Low	1	205	35.06	11.28	0.16	5.99	9.9
	2	212	36.03	11.1			5.8
Hatchery Control	3	421	33.01	11.16	0.32	11.35	6.5
	4	422	33.72	11.94			6.7
Hatchery High	5	424	26.07	9.78	1.41	35.66	5.8
	6	421	25.2	8.57			6.5
Semi-natural Low	7	85	16.92	7.89	NA	0.33	81.2
	8	98	11.29	5.63			78.3
Semi-natural High	9	861	13.79	6.02	NA	2.8	36.3
	10	839	12.01	5.37			37.9

Table 3: Parameter estimates of the full model for the linear mixed model investigating log weight variation.

Covariate	Fixed effects	Parameter estimate	Std. error	t value	P value	Overall p value
Treatment	Intercept	1.64	0.05	35.51	0.00	0.00
	Hatchery Control	-0.02	0.06	-0.35	0.74	
	Hatchery High	-0.12	0.06	-2.01	0.09	
	Semi-natural High	-0.45	0.06	-7.12	0.00	
	Semi-natural Low	-0.401	0.07	-5.92	0.00	
Group	Hybrid	-0.07	0.04	-1.99	0.07	0.00

	Wild	-0.27	0.04	-7.60	0.00	
Egg size	Egg size	-0.02	0.03	-0.68	0.51	0.00
Treatment * Group	Hatchery Control * Hybrid	-0.01	0.03	-0.25	0.80	
	Hatchery High * Hybrid	-0.01	0.03	-0.16	0.87	
	Semi-natural High * Hybrid	0.00	0.04	-0.01	0.99	
	Semi-natural Low * Hybrid	0.03	0.06	0.45	0.66	0.58
	Hatchery Control * Wild	-0.03	0.03	-1.03	0.32	
	Hatchery High * Wild	-0.09	0.04	-2.31	0.04	
	Semi-natural High * Wild	-0.05	0.05	-1.11	0.29	
	Semi-natural Low * Wild	-0.05	0.06	-0.90	0.39	
Treatment * Eggsize	Hatchery Control * Eggsize	0.01	0.01	1.07	0.30	
	Hatchery High * Eggsize	0.03	0.02	2.22	0.05	
	Semi-natural High * Eggsize	0.07	0.02	3.40	0.01	0.03
	Semi-natural Low * Eggsize	0.07	0.02	2.91	0.01	
Group * Eggsize	Hybrid * Eggsize	0.05	0.04	1.27	0.24	0.48
	Wild * Eggsize	0.30	0.03	0.90	0.39	
Random effects		Variance	Std. dev			
Family	Hatchery Low	0.002	0.044			
	Hatchery Control	0.001	0.025			
	Hatchery High	0.002	0.041			
	Semi-natural High	0.004	0.065			
	Semi-natural Low	0.004	0.062			
Tank		0.003	0.055			
Residual		0.020	0.150			

Table 4: Relative weight and log weight differences between each group within each treatment.

	Treatment	Origin	W (g)	Relative W (g) difference		Log W	Relative Log W difference	
				to Wild	to Hybrid		to Wild	to Hybrid
Hatchery	Low	Farm	45.2	1.8	1.2	1.64	1.2	1.1
		Hybrid	36.45	1.5		1.55	1.1	
		Wild	24.74			1.37		
	Control	Farm	42.95	1.9	1.2	1.62	1.2	1.1
		Hybrid	34.53	1.5		1.52	1.2	
		Wild	22.71			1.32		
	High	Farm	33.51	2	1.2	1.51	1.3	1.1
		Hybrid	26.85	1.6		1.41	1.2	
		Wild	16.68			1.19		
Semi-natural	Low	Farm	19	2	1.3	1.24	1.3	1.1
		Hybrid	15.04	1.6		1.13	1.2	
		Wild	9.3			0.92		
	High	Farm	16.68	1.9	1.3	1.19	1.3	1.1
		Hybrid	13.15	1.5		1.08	1.2	
		Wild	8.99			0.92		

Table 5: Parameter estimates of the glmm model investigating variation in survival and overall p values of each model covariate.

Covariate	Fixed effects	Parameter estimate	Std. error	Z value	P value	Overall p value
Treatment	Intercept	2.46	0.33	7.56	0.00	0.00
	Hatchery High	0.18	0.31	0.59	0.55	
	Hatchery Low	-0.73	0.36	-0.20	0.84	
	Semi-natural High	-1.97	0.22	-8.79	<2e-16	
	Semi-natural Low	-4.40	0.27	-16.13	<2e-16	
Group	Hybrid	1.08	0.52	2.08	0.04	0.59
	Wild	-0.03	0.46	-0.07	0.95	
Egg size	Egg size	-4.90	80.79	-0.06	0.95	0.86
Treatment * Group	Hatchey High * Hybrid	-0.19	0.51	-0.38	0.71	0.08
	Hatchery Low * Hybrid	0.21	0.66	0.32	0.75	
	Semi-natural High * Hybrid	-0.78	0.38	-2.06	0.04	
	Semi-natural Low * Hybrid	-0.43	0.43	-1.01	0.31	
	Hatchey High * Wild	0.13	0.45	0.28	0.78	
	Hatchery Low * Wild	-0.27	0.51	-0.53	0.60	
	Semi-natural High * Wild	-0.07	0.33	-0.22	0.83	
	Semi-natural Low * Wild	0.75	0.38	1.97	0.05	
Treatment * Eggsize	Hatchery High * Eggsize	53.54	31.76	1.69	0.09	0.00
	Hatchery Low* Eggsize	-20.76	32.41	-0.64	0.52	
	Semi-natural High * Eggsize	-4.02	21.54	-0.19	0.85	
	Semi-natural Low * Eggsize	-84.36	25.48	-3.30	0.00	
Group * Eggsize	Hybrid * Eggsize	74.19	107.60	0.69	0.49	0.89
	Wild * Eggsize	47.56	82.28	0.58	0.56	
Random effects		Variance	Std. dev			
	Tank	0.00	0.00			
	Family	0.24	0.49			
	Deviance	5331.30				

Figure 1

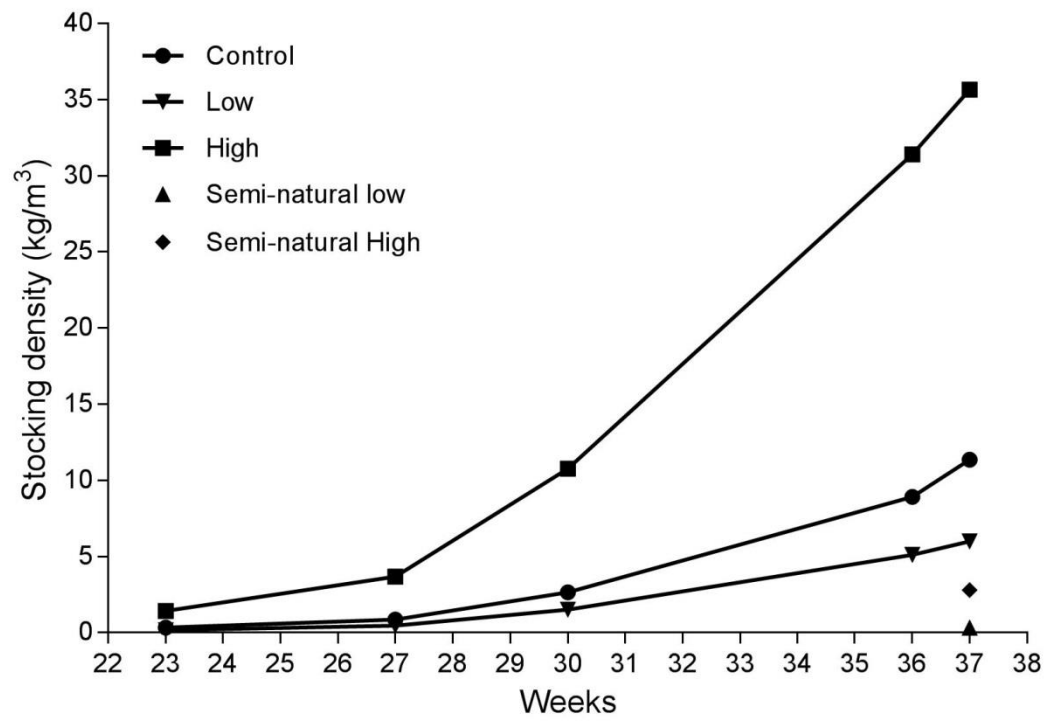


Figure 2

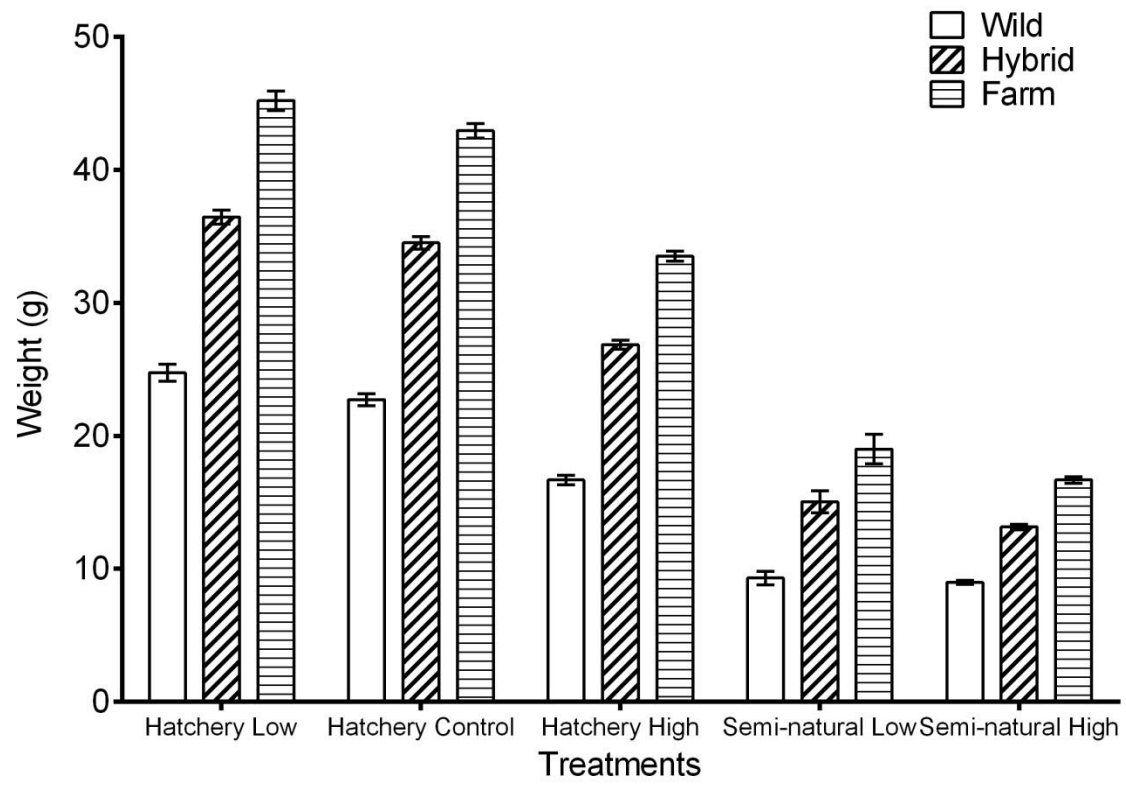


Figure 3

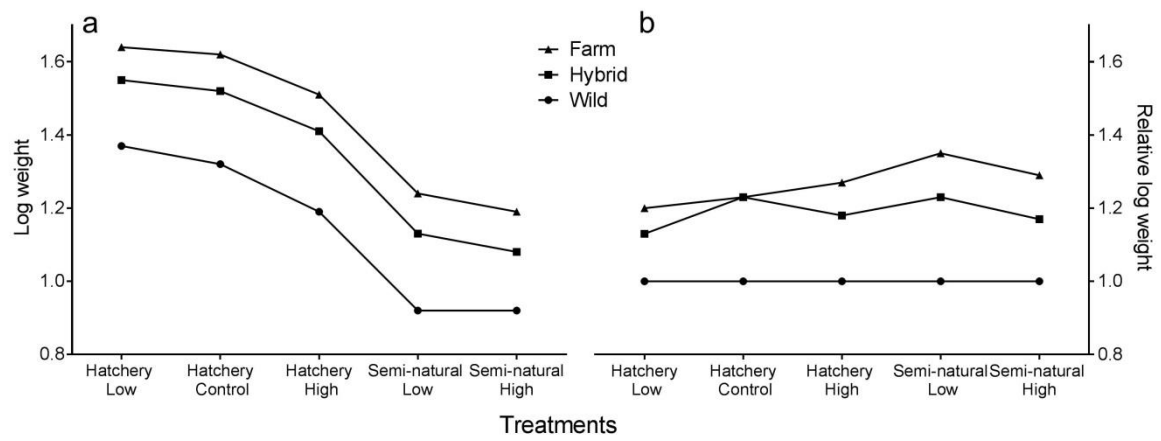
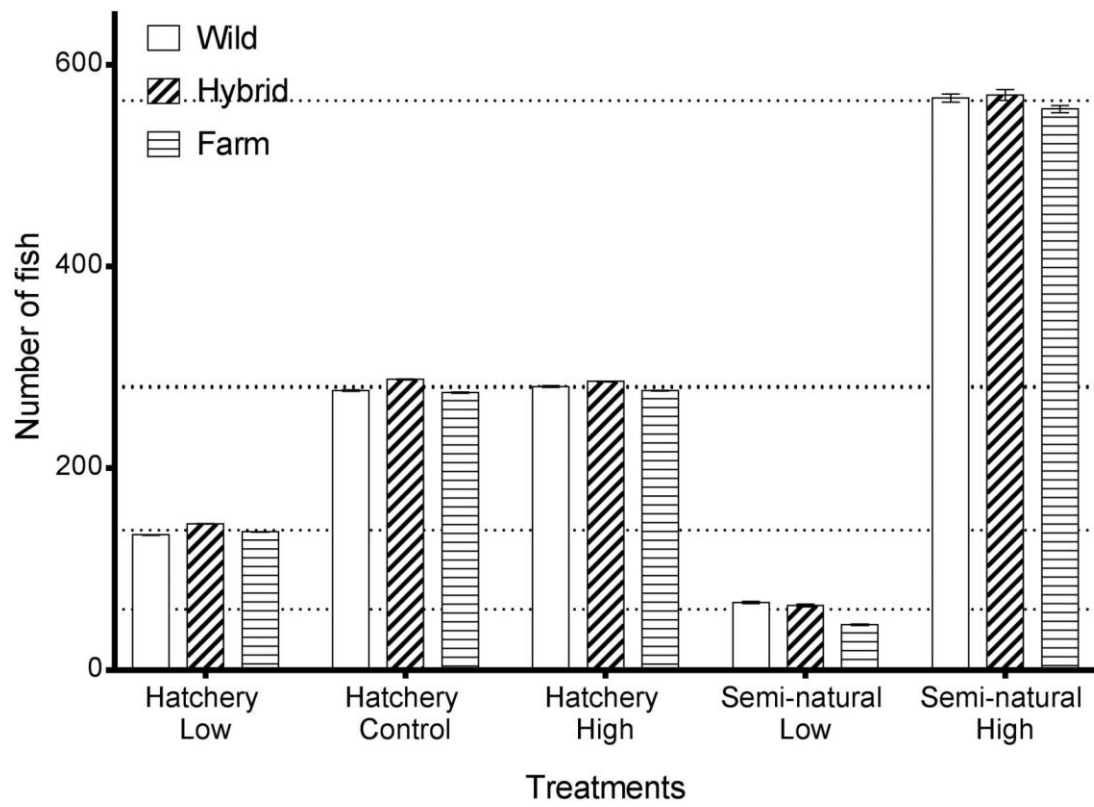


Figure 4



SUPPLEMENTARY TABLES AND FIGURES

Table S1: Family crosses for the experiment.

Family	Dam	Sire	Group
1	M1	M9	Farm
2	M1	E11	Hybrid
3	M2	M10	Farm
4	M2	E12	Hybrid
5	M3	M11	Farm
6	M3	E13	Hybrid
7	M4	M12	Farm
8	M4	E14	Hybrid
13	M7	M15	Farm
14	M7	E17	Hybrid
17	E1	E11	Wild
18	E2	E12	Wild
19	E3	E13	Wild
20	E4	E14	Wild
23	E7	E17	Wild

Table S2: Details of the microsatellite multiplexes used to assign the un-assignable individuals back to family.

Multiplex	Primers	Dye	Allele Size	No. Alleles	Direction	Sequences	References
1	SSsp2210	6FAM	124-176	14	F	AAG TAT TCA TGC ACA CAC ATT CAC TGC	Paterson et al. 2004
					R	CAA GAC CCT TTT TCC AAT GGG ATT C	
	SSspG7	PET	119-207	22	F	CTT GGT CCC GTT CTT ACG ACA ACC	Patterson et al. 2004
					R	TGC ACG CTG CTT GGT CCT TG	
	SsaD144	NED	102-254	37	F	TTG TGA AGG GGC TGA CTA AC	King et.al 2005
					R	TCA ATT GTT GGG TGC ACA TAG	
	Ssa202	6FAM	230-298	18	F	CTT GGA ATA TCT AGA ATA TGG C	O'Reilly et al. 1996
					R	GTT CAT GTG TTA ATG TTG CGT G	
	Sp2201	PET	227-367	33	F	TTA GAT GGT GGG ATA CTG GGA GGC	Patersson et al. 2004
					R	CGG GAG CCC CAT AAC CCT ACT AAT AAC	
	SsaD157	NED	271-411	35	F	ATC GAA ATG GAA CTT TTG AAT G	King et.al 2005
					R	GCT TAG GGC TGA GAG AGG AAT AC	
2	Ssa289	PET	112-134	10	F	CTT TAC AAA TAG ACA GAC T	McConnell et al. 1995
					R	GTC ATA CAG TCA CTA TCA TC	
	Ssa14	NED	134-146	6	F	CCT TTT GAC AGA TTT AGG ATT TC	McConnell et al. 1995
					R	CAA ACC AAA CAT ACC TAA AGC C	
	Ssa171	NED	197-255	26	F	TTA TTA TCC AAA GGG GTC AAA A	O'Reilly et al. 1996
					R	GAG GTC GCT GGG GTT TAC TAT	
	Sp2216	6FAM	190-270	21	F	GGC CCA GAC AGA TAA ACA AAC ACG C	Paterson et al. 2004
					R	GCC AAC AGC AGC ATC TAC ACC CAG	
	Sp1605	PET	216-268	22	F	CGT AAT GGA AGT CAG TGG ACT GG	Paterson et al. 2004
					R	CTG ATT TAG CTT TTT AGT GCC CAA TGC	

Table S3: Pair-wise comparisons of log weight conducted between groups and between treatments, and of the average egg size among groups. The p values have been adjusted for multiple comparisons using a Tukey adjustment. The Significance column denotes the p values as significance codes whereby ‘***’ <0.0001, ‘**’ <0.001, ‘*’ <0.01, ‘.’ ≤0.5 and ‘ns’ denotes not significantly different.

Contrast	Estimate	Std. Error	z value	P value	Significance
Hybrid - Farm	-0.09138	0.02071	-4.413	3.62e-05	***
Wild - Farm	-0.33787	0.02216	-15.245	1.00e-05	***
Wild - Hybrid	-0.24649	0.02254	-10.936	1.00e-05	***
Hatchery Control - Hatchery Low	-0.03282	0.05549	-0.591	0.9764	ns
Hatchery High - Hatchery Low	-0.15115	0.05672	-2.665	0.0593	ns
Semi-natural Low - Hatchery Low	-0.40929	0.0585	-6.997	<0.001	***
Semi-natural High - Hatchery Low	-0.4667	0.05722	-8.156	<0.001	***
Hatchery High - Hatchery Control	-0.11833	0.05605	-2.111	0.215	ns
Semi-natural Low - Hatchery Control	-0.37647	0.05844	-6.442	<0.001	***
Semi-natural High - Hatchery Control	-0.43388	0.05613	-7.729	<0.001	***
Semi-natural Low - Hatchery High	-0.25814	0.05718	-4.515	<0.001	***
Semi-natural High - Hatchery High	-0.31554	0.05655	-5.58	<0.001	***
Semi-natural High - Semi-natural Low	-0.0574	0.0577	-0.995	0.8577	ns
Hybrid egg size – Farm egg size	-0.00063	0.00021	-2.96	0.0087	**
Wild egg size – Farm egg size	0.0056	0.00021	26.28	<1e-04	***
Wild egg size – Hybrid egg size	0.0063	0.00021	29.61	<1e-04	***

Table S4: Full models investigating relationship between weight and egg size variation at the different treatments. The variables in bold were retained in the final models for each treatment. Egg size is only retained in the semi-natural and hatchery high treatments.

Model	N	Response	Random effects				Fixed effects					
			Variable	Chi.sq	Chi Df	P	Variable	Sum Sq	Num Df	Den Df	F	P
Hatchery Low	416	Log Weight	Family	243.58	1	<0.00	Egg size	0.016	1	12.97	1.24	0.29
							Group	1.10	2	11.94	43.08	0
Hatchery Control	840	Log Weight	Family	434.43	1	<0.00	Egg size	0.013	1	11.16	1.80	0.21
							Group	1.57	2	12.04	38.76	0
Hatchery High	844	Log Weight	Family	559	1	<0.00	Egg size	0.23	1	11.40	12.35	0.005
							Group	3.36	2	11.21	88.81	0
Semi-natural Low	181	Log Weight	Family	1.1	1	0.29	Egg size	0.86	1	NA	21	<0.00
							Group	3.47	2	NA	42.39	<0.00
Semi-natural High		Log Weight	Family	783.94	1	<0.00	Egg size	0.61	1	11.2	22.13	0.00006
							Group	2.35	2	11	42.74	0

Table S5: Full models investigating relationship between survival and egg size variation at the different treatments. Egg size is only retained in the hatchery high density treatment.

Model	Variable	Estimate	SE	Z	<i>P value</i>
Hatchery Low	Intercept	2.5	0.18	14.04	<2e-06
	Egg size	-0.006	0.18	-0.035	0.97
Hatchery Control	Intercept	2.8	0.22	12.6	<2e-06
	Egg size	0.19	0.21	0.89	0.37
Hatchery High	Intercept	2.94	0.2	14.25	<2e-06
	Egg size	0.64	0.22	2.95	0.003
Semi-natural Low	Intercept	0.58	0.17	3.48	0.0005
	Egg size	0.17	0.17	1.01	0.29
Semi-natural High	Intercept	-1.48	0.17	-8.75	<2e-06
	Egg size	-0.19	0.17	-1.14	0.254

Figure S1: Average monthly water temperature for the indoor hatchery tanks and outdoor semi-natural tanks during the experimental period. Water temperature was recorded daily and is presented as monthly mean + range.

